Description

# Monocotyledon plant cells and plants which synthesise modified starch

The present invention relates to monocotyledon plant cells and plants which are genetically modified, wherein the genetic modification consists of the introduction of an extraneous nucleic acid molecule which codes for a protein with the biological activity of an R1 protein. The present invention further relates to means and methods for the production thereof. Plant cells and plants of this type synthesise a modified starch which is characterised in that it has an increased phosphate content and/or a modified phoshorylation pattern and/or an increased final viscosity in an RVA profile and/or a reduced peak temperature in DSC analysis and/or an increased gel strength in the texture analysis compared with starch from corresponding non-genetically modified monocotyledon plants. Therefore, the present invention also relates to the starch which is synthesised from the plant cells and plants according to the invention, and to methods of producing said starch. The present invention further relates to wheat flours which contain said modified starches, and to food products and bakery products which contain said wheat flours and/or starch.

With regard to the increasing importance which has recently been attached to substances of plant content as renewable sources of raw materials, one of the tasks of biotechnological research is to endeavour to adapt these plant raw materials to the requirements of the industry which processes them. To facilitate the use of renewable raw materials in as many fields of use as possible, it is also necessary to provide a considerable multiplicity of substances.

Apart from oils, fats and proteins, polysaccharides constitute the important renewable raw materials from plants. In addition to cellulose, starch, which is one of

the most important storage materials in higher plants, assumes a central position in polysaccharides.

Polysaccharide starch is a polymer of chemically uniform basic components, namely glucose molecules. However, it is a very complex mixture of different forms of molecules, which differ with regard to their degree of polymerisation and the occurrence of branched region in the glucose chains. Starch therefore does not constitute a uniform raw material. A distinction is made between two chemically different components of starch: amylose and amylopectin. In typical plants which are used for starch production, such as maize, wheat or potatoes, the synthesised starch consists of up to about 20% - 30% of amylose starch and up to about 70% - 80% of amylopectin starch.

Amylose was for a long time considered to be a linear polymer consisting of α-1,4-glycosidically bonded α-D-glucose monomers. In more recent studies, however, the presence of about 0.1% of α-1,6-glycosidic branching sites has been detected (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92). Basically, however, achieving a complete separation of amylose from amylopectin very difficult, so that the quality of the amylose is strongly dependent on the type of separation method selected. In contrast to amylose, amylopectin is more strongly branched and comprises about 4% of branching sites which are formed due to the occurrence of additional α-1,6-glycoside linkages. Amylopectin constitutes a complexes mixture of differently branched glucose chains. A further significant difference between these two molecules is their molecular weight. Whereas amylose, depending on the origin of the starch, has a molecular weight of $5x10^5 - 10^6$ Da, that of amylopectin ranges between $10^7$ and $10^8$ Da. These two macromolecules can be by distinguished by their molecular weight and by their different physicochemical properties, which are most readily manifested by their different iodine bonding properties.

A further significant difference between amylose and amylopectin is the relative amounts of trace substances which can exist in association with these macromolecules. Amylose has a high affinity for hydrophobic molecules. In cereals in particular, amylose can be complexed with relatively high amounts of lipids (Morrison, Cereal Foods World 40, (1995), 437-446). On the other hand,

amylopectin can contain covalently bonded inorganic phosphate in the form of starch phosphate monoesters, which has not hitherto been described for amylose. High contents of phosphate monoesters are found in particular in starches which are obtained from tubers. Amongst commercially available starches, potato starch has the highest phosphate content, which can range between 10-30 nmol $mg^{-1}$ starch In some types of Curcuma the phosphate content can even be 2 to 4 times higher (Bay-Smidt et al., 5th ISPMP Meeting Proceedings, (1997), 741), whilst it is about 100 times less in cereals (Kasemsuwan and Jane, Cereal Chem. 73, (1996), 702-707). In contrast to starches from tubers, roots and legumes, the detectable phosphate in cereal starches (monocotyledon plants) rarely occurs in the form of starch monoester derivatives, but mainly occurs in the form of phospholipids (Jane et al., Cereal Foods World 41, (1996), 827-832).

Apart from its amylose/amylopectin ratio and phosphate content, the functional properties of starch are influenced by its molecular weight, its pattern of side chain distribution, its content of ions, its lipid and protein content, etc. Important functional properties which should be cited here are the solubility, the retrogradation behaviour, the water absorption capacity, the film-forming properties, the viscosity, the conglutination properties, the freeze-thaw stability, the stability in relation to acids, the gel strength, etc. The starch grain size can also be important for various applications.

In principle, the phosphate content can be modified either by genetic engineering approaches or by the subsequent chemical phoshorylation of native starches (see, for example: Starch Chemistry and Technology. Eds. R. L. Whistler, J. N. BeMiller and E. F. Paschall. Academic Press, New York, 1988, 349-364). Chemical modifications are generally costly and time-consuming, however, and result in starches, the physicochemical properties of which can differ from those of starches modified in vivo.

Since starches from monocotyledon wild-type plants, particularly from cereal plants (wheat, rice, maize, oats, millet, rye), only have a very low content of phosphate in the form of starch phosphate monoesters (Lim et al. Cereal Chem. 71, (1994), 488), one object of the present invention is to provide monocotyledon plants which

synthesise starches with an increased phosphate content (content of starch phosphate monoesters) and modified physicochemical properties compared with corresponding wild-type plant cells and plants.

The underlying object of the present invention is thus to provide genetically modified monocotyledon plant cells and plants which synthesise starches with modified structural and/or functional properties compared with corresponding non-genetically modified wild-type plant cells and plants, and is also to provide starch, the structural and/or functional properties of which differ from those of starch from corresponding non-genetically modified wild-type plant cells and plants and from those of chemically modified starch, and which is thus more suitable for general and/or special industrial purposes of use.

This object is achieved by the provision of the embodiments described in the claims, because it has surprisingly been found that the introduction of an extraneous nucleic acid molecule into the genome of monocotyledon plant cells and plants results in a modification of the structural and/or functional properties of the starch which is synthesised in said monocotyledon plant cells and plants.

Expression of the extraneous nucleic acid molecule is primarily advantageous in starch-storing organs of monocotyledon plants, particularly of wheat plants, and leads to an increase of the phosphate content and modification of the viscosity properties of the starch which can be isolated from the starch-storing organs compared with starches which can be isolated from starch-storing organs of corresponding non-genetically modified wild-type plants, particularly wheat plants. Moreover, the starches according to the invention are distinguished from chemically phosphorylated starches by a modified phoshorylation pattern and modified viscosity properties, and after conglutination of the starches and gel formation are also distinguished by modified gel strengths.

Thus the present invention relates to monocotyledon genetically modified plant cells, wherein the genetic modification consists of the introduction of at least one

extraneous nucleic acid molecule and the extraneous nucleic acid molecule is selected from the group consisting of:

a) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;

b) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;

c) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and

d) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (a), (b) or (c).

In the sense of the present invention, the term "genetically modified" means that the genetic information of the plant cell is modified by the introduction of an extraneous nucleic acid molecule and that the presence or expression of the extraneous nucleic acid molecule results in a phenotypic modification. The term "phenotypic modification" preferably means a measurable modification of one or more functions of the cells. For example, the genetically modified plant cells according to the invention exhibit a modified expression pattern. In the sense of the present invention, the term "genetically modified" means that the monocotyledon plant cell according to the invention contains at least one extraneous nucleic acid molecule which is integrated in the genome in a stable manner.

In the sense of the present invention, the term "extraneous nucleic acid molecule" is to be understood to mean a nucleic acid molecule which codes for a protein with the biological activity of an R1 protein, preferably an R1 protein from Solanum tuberosum, and which does not occur naturally in corresponding non-genetically modified wild-type plant cells. The extraneous nucleic acid molecule is preferably a recombinant molecule which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in plant cells. The monocotyledon plant cells according to the invention contain at least one extraneous nucleic acid molecule, wherein the latter is preferably linked to regulatory DNA elements which ensure transcription in plant cells, particularly with a promoter.

An example of an extraneous nucleic acid molecule which codes for an R1 protein from Solanum tuberosum is represented in SEQ ID No.1. Nucleotide sequences which code for an R1 protein from Solanum tuberosum have also been described in WO 97/11188 A1 and by Lorberth et al. (Nature Biotech. 16, (1998), 473-477).

The important characteristics of of R1 proteins from Solanum tuberosum are i) their amino acid sequence (see SEQ ID No. 2, for example); ii) their location in the stroma of plastids of plant cells, wherein they can exist there both in a form bound to starch grains and in soluble form; and iii) their capacity for influencing the degree of phoshorylation of starch in plants. For example, inhibition of the R1 gene which codes for an R1 protein from potatoes in transgenic potato plants results in a reduction of the phosphate content of the starch which can be isolated from the potato tubers. Furthermore, Lorberth et al. showed that the R1 protein from Solanum tuberosum is capable of phosphorylating bacterial glycogen if the corresponding R1 cDNA is expressed in E. coli (Lorberth et al., Nature Biotech. 16, (1998), 473-477). Ritte et al. (Plant J. 21, (2000), 387-391) showed that the R1 protein from Solanum tuberosum binds reversibly to starch grains in potato plants, wherein the strength of binding to the starch grain depends on the metabolic status of the plant. In starch grain-bound form, the protein in potato plants mainly occurs in leaves which are kept in the dark. After the leaves are illuminated, however, the protein is mainly present in a soluble form which is not bound to starch grains.

Moreover, inhibiting the expression of the R1 gene from potatoes in transgenic potato plants results in a "starch-excess" phenotype, i.e. the leaves of corresponding plants have an increased content of starch (Lorberth et al., Nature Biotech. 16, (1998), 473-477). In addition, the tubers of such potato plants are distinguished in that after cold storage they exhibit reduced “cold-induced sweetening” (Lorberth et al., Nature Biotech. 16, (1998), 473-477).

In principle, the extraneous nucleic acid molecule which codes for an R1 protein can originate from any kind of potato or potato plant, preferably from potatoes of the Tomensa, Desiree, Tempora and Thomana varieties.

In one preferred embodiment, the extraneous nucleic acid molecule has the nucleotide sequence represented in SEQ ID No. 1.

In a further preferred embodiment the invention comprises the extraneous nucleic acid molecule of the coding region of the nucleotide sequence represented in SEQ ID No.1.

In a further preferred embodiment of the invention, the extraneous nucleic acid molecule encodes an R1 protein from Solanum tuberosum which has the amino acid sequence given in SEQ ID No. 2.

In yet another embodiment of the invention, the extraneous nucleic acid molecules comprise the coding region of the mature (without a plastidary signal peptide) protein (bp 447-bp 4607 of the nucleotide sequence given in SEQ ID No. 1). Instead of the plastidary N-terminal signal peptide of the R1 protein from potatoes (amino acids 1 to 77 coded by nucleotides 216 to 446 of the nucleotide sequence given in SEQ ID No.1), in this embodiment of the invention the extraneous nucleic acid molecules comprise a heterologous plastidary signal sequence, i.e. a signal sequence which does not exist naturally in association with the mature R1 protein. Plastidary signal sequences are known to one skilled in the art.
The signal sequence of Ferrodoxin:$NADP^+$ oxidoreductase (FNR) from spinate can be used as a plastidary signal sequence, for example. This sequence contains the 5'-non-translated region as well as the flanking transit peptide sequence of the cDNA of the plastidary protein Ferrodoxin:$NADP^+$ oxidoreductase from spinate (nucleotides -171 to + 165; Jansen et al., Current Genetics 13, (1988), 517-522).

In addition, for example, the transit peptide of the waxy protein from maize plus the first 34 amino acids of the mature waxy protein can be used as a signal sequence (Klösgen et al., Mol. Gen. Genet. 217, (1989), 155-161). Furthermore, the transit peptide of the waxy protein from maize can be used without the first 34 amino acids of the mature waxy protein.

In a further preferred embodiment of the invention, the extraneous nucleic acid molecule constitutes a derivative of the nucleotide sequence given in SEQ ID No.1.

In the sense of the present invention, the expression "derivative" means that the sequences of these molecules differ at one or more positions from the nucleotide sequence given in SEQ ID No. 1 and exhibit a high degree of homology with the coding region of the nucleotide sequence given in SEQ ID No. 1. In addition, derivatives are distinguished in that they code for a protein with the biological activity of an R1 protein, preferably of an R1 protein from Solanum tuberosum.
In the sense of the present invention, "homology" means a sequence identity of nucleotide sequences of at least 65 %, particularly of at least 85 %, more particularly an identity of at least 90 %, preferably more than 95 % and most preferably more than 98 %. Differences from the nucleic acid molecules described above can occur due to deletion, addition, substitution, insertion or recombination.

The degree of homology is preferably determined by comparing the sequence of the respective nucleotide sequence with the coding region of SEQ ID No.1, particularly with region of SEQ ID No.1 (bp447-bp4607) which codes for the mature protein of the nucleotide sequence given in SEQ ID No. 1). If the sequences to be compared are of different lengths, the degree of homology preferably refers to the percentage of nucleotides in the shorter nucleotide sequence which are identical to the nucleotides of the longer sequence, i.e. the sequence identity is determined for the region in which the respective nucleotide sequences overlap. Sequence comparisons can be made using known computer programs, such as the ClustalW program (Thompson et al., Nucleic Acids Research 22, (1994), 4673-4680), which is distributed by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE; European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany). Clustal W can also be downloaded from various internet sites, e.g. that of the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and that of the EBI (European Bioinformatics Institute) (ftp://ftp.ebi.ac.uk/pub/software/), as well as from internet sites with links to the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).
When using the ClustalW program (Version 1.8), the default values of the various parameters are employed. In the case of DNA sequence comparisons, these

parameters have the following values: KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

In the case of protein sequence comparisons the default values of the parameters of the ClustalW program are likewise used. These parameters have the following values: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

The degree of homology can be determined, for example, using known computer programs such as Mview (http://www.sacseeucsf.edu/documentation/seqsoftware/mview; Brown, N.P., Leroy C., Sander C. (1998). MView: A Web compatible database search or multiple alignment viewer. *Bioinformatics*,14(4):380-381).

In the sense of the present invention, "homology" also means that there is functional and/or structural equivalence between the respective nucleic acid molecules or the proteins which they encode. The nucleic acid molecules which are homologous with the nucleic acid molecules described in SEQ ID No.1 and which constitute derivatives of these molecules are variations of these molecules which constitute modifications which perform the same biological function, and which in vivo are also capable of increasing the phosphate content of starches in monocotyledon plants. These can be either naturally occurring variations, for example sequences from other types of potatoes, or mutations, wherein said mutations may have been formed naturally or may have been introduced by targeted mutagenesis.

In a further embodiment of the invention, the derivative of SEQ ID No.1 comprises allelic variants of the nucleic acid molecule given in SEQ ID No.1.
These allelic variants are naturally occurring variants which are capable of increasing the phosphate content of starches.

In a further embodiment of the invention, the extraneous nucleic acid molecule constitutes a fragment of the extraneous nucleic acid molecule which is defined according to the invention.

In the sense of the present invention, the term "fragment" denotes parts of the extraneous nucleic acid molecule which code for a biologically active, preferably enzymatically active, part of the R1 protein.

A biologically active part of an R1 protein is distinguished in that *in planta* it can cause an increase of the phosphate content with superexpression of the extraneous nucleic acid molecule and/or that after expression in E. coli it facilitates phoshorylation of glycogen (see WO 97/11188 A1, Example 9).

The proteins which are encoded by the different variants (fragments, derivatives, allelic variants) of the extraneous nucleic acid molecule exhibit defined general characteristics. Examples of the latter include the biological activity, the enzymatic activity, or a similar primary structure which can be investigated by means of the protein homology comparisons described above. The amino acid sequences of the proteins exhibit a mutual homology of at least 60%, preferably at least 85%, particularly at least 95% and most preferably at least 97%.

In addition to the characteristics described above, the extraneous nucleic acid molecules may be characterised in that they code for proteins with the biological activity of R1 proteins, which comprise at least one, preferably at least five, particularly at least 10 and most preferably all of the following characteristic peptide motifs:
DKAAET (SEQ ID No. 3), IADME (SEQ ID No. 4), VWMRFM (SEQ ID No. 5), MQEWHQ (SEQ ID No. 6), LGHYM (SEQ ID No. 7), ERGYEE (SEQ ID No.8), KAVLDR (SEQ ID No. 9), LSSLL (SEQ ID No. 10), IPDGAV (SEQ ID No.11), KVCFAT (SEQ ID No. 12), ISADEF (SEQ ID No. 13), PFGVFE (SEQ ID No. 14), SSGDD (SEQ ID No. 15), SFICKK (SEQ ID No. 16).

Amongst other differences, the plant cells according to the invention can also be distinguished from naturally occurring plant cells in that they contain an extraneous nucleic acid molecule which does not occur naturally in these cells, or that a molecule such as this is integrated at a place in the genome of the cell at which it does not occur naturally, i.e. in a different genomic environment. Moreover,

transgenic plant cells of this type according to the invention can be distinguished from naturally occurring plant cells in that they contain at least one copy of the extraneous nucleic acid molecule which is integrated in a stable manner in their genome contain, optionally in addition to copies of a molecule such as this which occur naturally in the cells. If the nucleic acid molecules which are introduced into the cells are additional copies of molecules which already occur naturally in the cells, the plant cells according to the invention can be distinguished in particular from naturally occurring plant cells in that these additional copies are located at sites in the genome at which they do not occur naturally. This can be detected subsequently by means of Southern blotting analysis, for example.

Furthermore, the plant cells according to the invention are preferably distinguished from naturally occurring plant cells by at least one of the following features: if the nucleic acid molecule which is introduced is heterologous with respect to the plant the transgenic plant cells according to the invention comprise transcripts of the nucleic acid molecules which are introduced, in fact even in those organs in which no transcripts can be detected in wild-type plants. Plant cells according to the invention preferably contain transcripts of the extraneous nucleic acid molecules. These can be detected by Northern blotting analysis, for example. Plant cells according to the invention preferably contain a protein which is encoded by an introduced extraneous nucleic acid molecule. This additional protein can be detected by immunological methods for example, particularly by Western blotting analysis.

In one preferred embodiment, the plant cells and plants according to the invention exhibit an increased biological activity of the R1 protein compared with corresponding non-genetically modified wild-type plant cells and wild-type-plants. In connection with the present invention, an "increased biological activity of the R1 protein" can be determined, for example, by measuring the phosphate content of the starches which are synthesised in the plant cells and plants according to the invention. Compared with starches from corresponding non-genetically modified wild-type plant cells, starches from the plant cells according to the invention, which exhibit increased biological activity of the R1 protein, are distinguished in that they synthesise a starch with an increased phosphate content, preferably an increased phosphate content in the C6 position of the glucose monomer.

Moreover, an increased biological activity of the R1 protein can be determined by measuring the amount of R1-transcripts, e.g. by Northern blotting analysis, compared with the amount of R1-transcripts of corresponding non-genetically modified wild-type plants.

Furthermore, an increased biological activity of the R1 protein can be determined by measuring the amount of R1 protein, e.g. by Western blotting analysis, compared with the amount of R1 protein of corresponding non-genetically modified wild-type plants.

It has been found that the monocotyledon plant cells according to the invention synthesise a starch with an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plant cells of wild-type-plants.

Therefore, the present invention preferably relates to plant cells according to the invention which synthesise a starch has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plant cells of wild-type-plants.

In the sense of the present invention, the term "phosphate content" denotes the content of phosphate which is covalently bonded in the form of starch phosphate monoesters.

In the sense of the present invention, the term "C6 position" is to be understood to mean phosphate groups, which are bonded to the glucose monomer of the starch at carbon atom position "6".

In connection with the present invention, the expression "increased phosphate content in the C6 position" is to be understood to mean that glucose monomer phosphate groups can be detected in the in the C6 position by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117, see methods).

The expression "increased phosphate content in the C6 position" is preferably understood to mean an increase in the phosphate content of the starch in the C6 position of the glucose monomer by at least 20%, preferably by at least 50% and most preferably by at least 100%, compared with the phosphate content of starch from corresponding non-genetically modified wild-type-plants.

In principle, the C2, C3 and C6 positions of the glucose units can be phosphorylated in the starch in vivo. In connection with the present invention, the phosphate content in the C6 position (= C6 P content) can be determined by a glucose-6-phosphate determination by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) (see methods).

In the sense of the present invention, the term "modified phoshorylation pattern means that the total phosphate content of phosphate groups which are covalently bonded to the starch within the various layers of the starch grain is modified compared with chemically phosphorylated starches which are produced from starches from corresponding non-genetically modified plants. Chemically phosphorylated starches are distinguished by a phosphate gradient inside the starch grains, wherein the outer layers are generally more strongly phosphorylated than are the inner layers. In contrast to this, the starches according to the invention are distinguished in that the phosphate groups are distributed differently over the various layers of the starch grain, i.e. the starches according to the invention can be characterised in that they do not comprise the gradients from the outside to the inside which are typical of chemically phosphorylated starches.

Methods of investigatingh the phoshorylation pattern of starch are known to one skilled in the art (see Jane and Shen, Carbohydrate Research 247, (1993), 279-290; Gough and Pybus, Staerke 25, (1973), 123-130, for example). These methods are based on step-wise chemical conglutination of the various layers of the starch grains, in which the conglutinated layers of the starch grains are mechanically removed in steps. This shelling procedure is followed by a determination of the total phosphate content of the various starch grain layers by standard methods.

In the sense of the present invention, the term "chemically phosphorylated starch" is to be understood to mean a starch which is produced by the chemical phoshorylation of native starch from corresponding non-genetically modified plant cells and/or plants. In connection with the present invention, chemically phosphorylated starch is preferably distinguished by an amylose content which is comparable with the amylose content of the starches according to the invention. During the chemical phoshorylation of native starches, the phosphate content in the C6 position is adjusted by selecting suitable test conditions so that the phosphate content in the C6 position of the chemically phosphorylated starch is identical to and/or comparable with the phosphate content in the C6 position of the glucose monomer of the starches according to the invention.

In connection with the present invention, the term "modified viscosity properties" is to be understood in particular to mean an increase in the final viscosity in an RVA profile and/or a lowering of the peak temperature in DSC analysis.

In a further preferred embodiment, the plant cells according to the invention synthesise a starch which has an increased final viscosity compared with starch from corresponding non-genetically modified wild-type plants.

In connection with the present invention, the term "final viscosity" is to be understood to mean the viscosity which can be determined from a viscosity profile (see Figure 1) and which is denoted there as "final viscosity = Fin". The viscosity profile can be obtained by means of a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd, Investment Support Group, Warriewood, NSW 2102, Australia). In the analysis of wheat starches, the viscosity profile is determined by the following procedure: 2.5 g starch (dry substance) are taken up in 25 ml $H_2O$ and used for analysis in a Rapid Visco Analyzer (Newport Scientific Pty Ltd., Investment Support Group, Warriewood NSW 2102, Australia). The instrument is operated according to the manufacturer's instructions. The complete temperature programme is illustrated schematically in Figure 1. Performing an RVA analysis is described in detail below (see methods).

In the sense of the present invention, the term "increased final viscosity" means that the final viscosity is increased by at least 10%, preferably by at least 30%, particularly by at least 50% and most preferably by at least 80% compared with starches from corresponding non-genetically modified wild-type plant cells, wherein the final viscosity can be increased by 1000% at most, preferably by 500% at most, particularly by 250% at most, compared with starches from corresponding non-genetically modified wild-type plant cells.

In a further preferred embodiment, the plant cells according to the invention synthesise a starch which has an identical or comparable phosphate content in the C6 position of the glucose monomer and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants and/or compared with chemically phosphorylated starches.

In the context of the present invention, the term "peak temperature" should be understood to mean the temperature Tp which can be determined by means of differential scanning calorimetry (DSC), which is known to one skilled in the art. The peak temperature is the temperature which can generally be assigned to the first peak maximum of the DSC curve. It can be determined, for example, using an instrument supplied by Perkin Elmer (instrument designation: DSC-7) using capsules of large volume, wherein the sample to be investigated comprises a ratio of starch to total water content of about 1:4 and measurements are made over a temperature range from 10°C to 160°C at a heating rate of 10°C/min (see Example 4).

The term "reduced peak temperature" Tp means that compared with starches from corresponding non-genetically modified wild-type plant cells the peak temperature Tp is reduced by at least 1.5°C, preferably by at least 2.5°C, particularly by at least 4°C and most preferably by at least 6°C, and is reduced at most by 5°C, by 12°C or by 9°C.

In a further preferred embodiment, the present invention relates to plant cells according to the invention which synthesise a starch which after conglutination

forms a gel, which has an increased gel strength compared with a gel formed from starch from corresponding non-genetically modified wild-type plant cells.

In the sense of the present invention, the term "increased gel strength" is to be understood to mean an increase gel strength by at least 20%, particularly by at least 50%, preferably by at least 80% and most preferably by at least 100%, with the maximum reduction being 500% at most or 250% at most compared with the gel strength of starch from corresponding non-genetically modified wild-type plant cells. In connection with the present invention, the gel strength can be determined by means of a texture analyser under the conditions described below (see methods).

In a further embodiment of the invention, the increase in the phosphate content of the starches can relate to the amylopectin component of the starch.
Therefore, the present invention also relates to plant cells according to the invention which synthesise a starch, the amylopectin component of which is phosphorylated and the amylose component of which has a reduced total phosphate content compared with the amylose component of corresponding chemically phosphorylated starches with the same starch phosphate content in the C6 position of the glucose monomer.
Therefore, in contrast to chemically phosphorylated starches with the same phosphate content in the C6 position of the glucose monomer, the starches of the plant cells according to the invention are distinguished in that that the amylose component of the starch from the plant cells according to the invention has a reduced total phosphate content and/or a reduced phosphate content in the C6 position of the glucose monomer compared with the amylose component of chemically phosphorylated starch with the same phosphate content in the C6 position of the glucose monomer, which is produced from starch, which preferably has a comparable amylose content, from corresponding non-genetically modified plants.

In connection with the present invention, the term "total phosphate content" is to be understood to mean the content of phosphate which is covalently bound in the form of starch phosphate monoesters in the C2, C3 and C6 positions of the glucose units.

According to the invention, the content of phosphorylated non-glucans, such as phospholipids; is not included under the term "total phosphate content". Phosphorylated non-glucans therefore have to be quantitatively separated before the determination of the total phosphate content.

Methods of separating phosphorylated non-glucans (e.g. phospholipids) from starch are known to one skilled in the art.

In the sense of the present invention, the term "reduced total phosphate content" is to be understood to mean a reduction of the total phosphate content by at least 5%, particularly by at least 20%, preferably by at least 50% and most preferably by at least 80%, compared with the total phosphate content of the amylose component of a chemically phosphorylated starch with the same C6 phosphate content which is produced from starch from corresponding non-genetically modified plants. Methods of determining of the total phosphate content are known to one skilled in the art and are described below (see methods).

In the sense of the present invention, the term "reduced phosphate content in the C6 position of the glucose monomer" should be understood to mean a reduction of the phosphate content in the C6 position of the glucose monomer of the amylose component by at least 10%, particularly by at least 20%, preferably by at least 50% and most preferably by at least 80% compared with the phosphate content in the C6 position of the glucose monomer of the amylose component of a chemically phosphorylated starch of the same C6 phosphate content which is produced from starch from corresponding non-genetically modified plants.

Methods of determining of the phosphate content in the C6 position of the glucose monomer are known to one skilled in the art and are described below (see methods).

In the sense of the present invention the term "amylopectin component" is to be understood to mean the amylopectin of the starch.

In the sense of the present invention, term "amylose component" is to be understood to mean the amylose of the starch.

Methods of separating amylose and amylopectin are known to one skilled in the art. For example, the amylose component can be obtained by aqueous leaching of the starch, as described, for example, by Roger & Colonna (International Journal of Biological Macromolecules 19, (1996), 51-61).

In a further preferred embodiment of the present invention, the starches according to the invention are additionally characterised in that the ratio of the C6 phosphate content of the glucose monomer in the C6 position of the amylose component to the C6 phosphate content of the glucose monomer in the C6 position of the starch is less than 0.75, particularly less than 0.5, preferably less than 0.25 and most preferably less than 0.20. In contrast to chemically phosphorylated starches, the starches according to the invention are distinguished in that the major part of the phosphate in the C6 position of the glucose monomer is detected in the amylopectin component of the starch and not in the amylose component.

The plant cells according to the invention originate from monocotyledon plants. They are preferably plant cells from plants which are used in agriculture, i.e. from plants, which are cultivated by humans for the purpose of nourishment or for technical purposes, particularly industrial purposes. Thus the present invention preferably relates to plant cells from starch-synthesising or starch-storing plants, e.g. rye, barley, oats, wheat, millet, rice or maize.

In one preferred embodiment of the present invention, the plant cells according to the invention originate from a plant of the group consisting of wheat, rice, barley, oats, rye and maize. Plant cells from wheat, rice and maize plants are preferred; plant cells from wheat plants are particularly preferred.

In one particularly preferred embodiment, the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P $mg^{-1}$ starch, particularly at least 0.5 nmol C6 P $mg^{-1}$, preferably at least 1 nmol C6 P $mg^{-1}$ starch, more preferably at least 2 nmol C6 P $mg^{-1}$ starch, most preferably at least 5 nmol C6 P $mg^{-1}$ starch,

most particularly preferably at least 10 nmol C6 P $mg^{-1}$ starch, wherein the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P $mg^{-1}$ starch at most, particularly 50 nmol C6 P $mg^{-1}$ starch at most, and most particularly 25 nmol C6 P $mg^{-1}$ starch at most.

In a further embodiment of the invention, the plant cells according to the invention can synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of at least 15 nmol C6 P $mg^{-1}$ starch, wherein the plant cells according to the invention synthesise a starch which in the C6 position of the glucose monomer have a phosphate content of 100 nmol C6 P $mg^{-1}$ starch at most, particularly 50 nmol C6 P $mg^{-1}$ starch at most, and most particularly 25 nmol C6 P $mg^{-1}$ starch at most.

In a further embodiment, the present invention relates to a method of producing a plant cell according to the invention, wherein a cell of a monocotyledon plant is genetically modified, and wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule.

There is a multiplicity of techniques which are available for the transfer of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a transformation agent, fusion of protoplasts, injection, electroporation of DNA, the introduction of DNA by means of biolistic techniques and other possible techniques. The use of the transformation of plant cells facilitated by agrobacteria has been intensively investigated and is satisfactorily described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and An et al. EMBO J. 4, (1985), 277-287. For the transformation of potatoes, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33.), for example.

The transformation of monocotyledon plants has in the meantime been routinely accomplished by means of a biolistic technique and by means of agrobacteria (Komari et al., (1998), Advances in cereal gene transfer; Current Opinion in Plant

Biotechnology 1, page 161 et seq.; Bilang et al. (1999), Transformation of Cereals, Genetic Engineering, 12, pages 113-148, edited by: JK Setlow, Kluwer Academic / Plenum Publisher, New York). Vectors based on agrobacteria have been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). Alternative systems for the transformation of monocotyledon plants include transformation by means of a biolistic technique (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells, and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described many times in the literature (see WO 95/06128, EP 0513849, EO 0465875, EP 292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726, for example).

The successful transformation of other types of cereals has also been described, e.g. for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Becker et al., Plant J. 5 (2), (1994), 229-307; Nehra et al., Plant J. 5, (1994), 285-297).

Various transformation methods have been described for rice, e.g. transformation facilitated by agrobacteria (Hiei et al., Plant J. 6 (1994), 271-282; Hiei et al., Plant Mol. Biol. 35 (1997), 205-218; Park et al., J. Plant Biol. 38 (1995), 365-371), protoplast transformation (Datta, In "Gene transfer to plants", Potrykus, Spangenberg (Eds.), Springer-Verlag, Berlin, Heidelberg, 1995, 66-75; Datta et al., Plant Mol. Biol. 20 (1992), 619-629; Sadasivam et al., Plant Cell Rep. 13 (1994), 394-396), a biolistic technique for plant transformation (Li et al., Plant Cell Rep. 12 (1993), 250-255; Cao et al., Plant Cell Rep. 11 (1992), 586-591; Christou, Plant Mol. Biol. (1997), 197-203), and electroporation (Xu et al., In "Gene transfer to plants", Potrykus, Spangenberg (Eds.), Springer-Verlag, Berlin, Heidelberg, 1995, 201-208).

The present invention further relates to plants containing the plant cells according to the invention and/or which can be produced by regeneration from plant cells according to the invention. These plants are preferably useful monocotyledon plants, e.g. rye, barley, oats, wheat, millet, rice and maize. Wheat, rice and maize are preferred; wheat is particularly preferred.

The plants according to the invention synthesise a modified starch, the phosphate content of which and/or the phoshorylation pattern of which and/or the viscosity properties of which differ from those of starches from corresponding non-genetically modified wild-type-plants, as described in connection with the plant cells according to the invention.

Therefore, the present invention also relates to plants according to the invention which synthesise a starch, which has an increased phosphate content in the C6 position of the glucose monomer and/or exhibits a modified phoshorylation pattern and/or modified viscosity properties compared with starch from corresponding non-genetically modified plants.
The statements made in connection with the plant cells according to the invention are applicable with regard to the increase of the phosphate content in the C6 position of the glucose monomer of the starch, the modification of the phoshorylation pattern and the modification of the viscosity properties of the starch.
The present invention preferably relates to plants according to the invention which synthesise a starch after conglutination forms a gel, which has an increased gel strength compared with a gel formed from starch from corresponding non-genetically modified wild-type-plants.

The term "increased gel strength" was defined in connection with the plant cells according to the invention.

In a further preferred embodiment of the invention, the plants according to the invention, preferably wheat plants, synthesise a starch, preferably wheat starch, which in the C6 position of the glucose monomer has a phosphate content of at least 0.1 nmol C6 P $mg^{-1}$starch, particularly at least 0.5 nmol C6 P $mg^{-1}$, preferably at

least 1 nmol C6 P $mg^{-1}$ starch, most preferably at least 2 nmol C6 P $mg^{-1}$ starch, particularly at least 5 nmol C6 P $mg^{-1}$ starch, quite most preferably at least 10 nmol C6 P $mg^{-1}$ starch, wherein the plants according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P $mg^{-1}$ starch at most, particularly 50 nmol C6 P $mg^{-1}$ starch at most, or 25 nmol C6 P $mg^{-1}$ starch at most.

In a further embodiment of the invention, the plants according to the invention can synthesise a starch which in the in the C6 position of the glucose monomer has a phosphate content of at least 15 nmol C6 P $mg^{-1}$ starch, wherein the plants according to the invention synthesise a starch which in the C6 position of the glucose monomer has a phosphate content of 100 nmol C6 P $mg^{-1}$ starch at most, particularly 50 nmol C6 P $mg^{-1}$ starch at most or 25 nmol C6 P $mg^{-1}$ starch at most.

In a further preferred embodiment of the invention, the plants according to the invention synthesise the starch according to the invention in the starch-storing organs of the plant according to the invention.

In a most preferred embodiment, the plants according to the invention synthesises a starch in its starch-storing organs which has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or modified viscosity properties compared with starch from starch-storing organs of corresponding non-genetically modified wild-type plants.

In this connection, the expression "starch-storing organs" is to be understood to mean those organs, e.g. the grains of maize, rice and wheat plants, which incorporate stored starch, in contrast to those organs, such as leaves, which only synthesise starch transiently.

Expression of the extraneous nucleic acid molecule is particularly advantageous in starch-storing organs of monocotyledon plants, especially in wheat plants, and results in an increase of the phosphate content of the starches which can be isolated from the starch-storing organs compared with starches which can be isolated from

the starch-storing organs of corresponding non-genetically modified wild-type plants, particularly wheat plants.

Expression of the extraneous nucleic acid molecule in starch-storing organs of monocotyledon plants can be achieved firstly by the use of constitutive promoters, e.g. the promoter of the 35S RNA of the cauliflower mosaic virus (see US-A-5,352,605, for example) and the ubiquitin promoter of maize (see US-A-5,614,399, for example).

Promoters are preferably used which are specific for starch-storing organs, e.g. endosperm-specific promoters, such as the glutelin promoter (Leisy et al., Plant Mol. Biol. 14, (1990), 41-50; Zheng et al., Plant J. 4, (1993), 357-366; Yoshihara et al., FEBS Lett. 383, (1996), 213-218), the shrunken-1 promoter (Werr et al., EMBO J. 4, (1985), 1373-1380), the HMW promoter of wheat (Anderson, Theoretical and Applied Genetics 96, (1998), 568-576, Thomas, Plant Cell 2 (12), (1990), 1171-1180), the USP promoter, the phaseolin promoter (Sengupta-Gopalan, Proc. Natl. Acad. Sci. USA 82 (1985), 3320-3324, Bustos, Plant Cell 1 (9) (1989), 839-853) or promoters of zein genes from maize (Pedersen et al., Cell 29, (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93) or caryopsis-specific promoters of GBSSI (granule bound starch synthase I) (DE10041861.9) and of SSII (soluble starch synthase II) from wheat (DE10032379.0).

In one particularly preferred embodiment, the plants according to the invention exhibit R1 gene expression in the starch-storing organs of said plants.

R1 gene expression can be determined, for example, by measuring the amount of R1 transcripts, e.g. by Northern blotting analysis, compared with the amount of R1 transcripts of wild-type plants.

Moreover, in connection with the present invention, R1 gene expression can be determined by measuring the amount of R1 protein, e.g. by Western blotting analysis. The amount of R1 protein is preferably determined by means of protein extracts which are isolated from plant cells of endosperm.

In a further embodiment of the invention, the plants according to the invention exhibit R1 gene expression in the starch-storing organs of said plants, which is increased compared with R1 gene expression in starch-storing organs of corresponding non-genetically modified wild-type-plants, preferably by at least 50%, most preferably by at least 100%, particularly by at least 250% and most particularly by at least 500%.

In one most preferred embodiment, the plants according to the invention exhibit a organ-specific expression of the extraneous nucleic acid molecule in the starch-storing organs of the plants according to the invention.

In the sense of the present invention, the term "organ-specific" is to be understood to mean that the selected promoter favours expression of the extraneous nucleic acid molecule in the starch-storing organs compared with mature leaves, and results in significantly increased degree of expression, such as a level of expression which is increased at least 2- to 5 times, preferably 5- to 10 times, most preferably 10- to 100 times.
In this embodiment of the invention, the plants according to the invention are distinguished in that due to the organ-specific expression of the extraneous nucleic acid molecule they exhibit increased R1 gene expression in the starch-storing organs compared with the gene expression of the endogeneous R1 gene in the starch-storing organs of a corresponding non-genetically modified wild-type-plant. In this embodiment of the invention, an increase in R1 gene expression in the leaves of the plants according to the invention cannot be detected to the same extent as that in the starch-storing organs. The increase in R1 gene expression is preferably related solely to the starch-storing organs.

In a preferred embodiment of the invention, the plants according to the invention are plants from the group consisting of wheat, rice, barley, millet, oats, rye and maize. Wheat, rice and maize plants are preferred; wheat plants are particularly preferred.

The invention also relates to a method of producing a plant as defined according to the invention, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule;

(b) a plant is regenerated from the cell according to step (a); and optionally

(c) further plants are produced from the plant produced according to step (b).

The present invention further relates to a method of producing a plant as defined according to the invention, which in the starch-storing organs of said plant synthesises a starch with an increased phosphate content and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from starch-storing organs of wild-type-plants, wherein

(a) a cell of a monocotyledon plant is genetically modified, wherein the genetic modification consists of the introduction of at least one extraneous nucleic acid molecule as defined according to the invention;

(b) a plant is regenerated from the cell according to step (a); and optionally

(c) further plants are produced from the plant produced according to step (b).

The same applies to the genetic modification which is introduced according to step (a) as that which has already been explained above in connection with the plant cells and plants according to the invention.

The regeneration of plants according to step (b) can be accomplished by methods known to one skilled in the art.

The production of further plants according to step (c) of the method according to the invention can be accomplished, for example, by vegetative propagation ) e.g. via cuttings, tubers or via callus cultivation and the regeneration of whole plants) or by sexual propagation. Sexual propagation preferably occurs in a controlled manner, i.e. selected plants with defined properties are crossed and propagated with each other.

In one preferred embodiment of the invention, the methods according to the invention are distinguished in that the extraneous nucleic acid molecule which is

introduced into the plant cell according to step (a) is selected from the group consisting of:

(i) nucleic acid molecules which comprise the coding region of the nucleotide sequence represented in Seq ID No. 1;

(ii) nucleic acid molecules which encode an R1 protein from Solanum tuberosum with the amino acid sequence given in Seq ID No. 2;

(iii) nucleic acid molecules which constitute a derivative of the nucleotide sequence given in Seq ID No. 1; and

(iv) nucleic acid molecules which constitute fragments of the nucleic acid molecules cited in (i), (ii) or (iii).

In one particularly preferred embodiment, the methods according to the invention are distinguished in that the extraneous nucleic acid molecule is under the control of a promoter, preferably an endosperm-specific and/or caryopsis-specific promoter, which organ-specifically facilitates R1 gene expression in starch-storing tissue.

Promoters of this type, such as endosperm-specific and caryopsis-specific promoters, have been exemplified above in connection with the plants according to the invention.

In a further embodiment, the methods according to the invention relate to useful monocotyledon plants, e.g. rye, barley, oats, wheat, millet, rice and maize. Wheat, rice and maize are preferred; wheat is particularly preferred.

The present invention also relates to the plants which can be obtained by the method according to the invention.

The present invention also relates to propagation material for plants containing plant cells according to the invention, and to the plants produced by the method according to the invention. In the sense of the present invention, the term "propagation material" comprises any constituents of the plant which are suitable for production or propagation by a vegetative or generative route. Cuttings, callus cultures, rhizomes or tubers are suitable for vegetative propagation, for example. Other propagation

material comprises fruit, seeds, seedlings, protoplasts, cell cultures etc., for example. Seeds are the preferred propagation material.

In one preferred embodiment, the present invention relates to maize grains.

In a most preferred embodiment, the present invention relates to wheat grains.

The maize and wheat grains according to the invention are particularly suitable for the production of fodder and of food products.

The present invention further relates to the use of extraneous nucleic acid molecules as defined according to the invention for the production of plants according to the invention, preferably of wheat, maize and rice plants, most preferably wheat plants, or for the production of monocotyledon plant cells according to the invention.

The plant cells and plant according to the invention synthesise, particularly in their starch-storing organs, a starch, the physicochemical properties of which, particularly the phosphate content of and/or the viscosity behaviour of which and/or the phoshorylation pattern of which is modified compared with that of starch synthesised in wild-type-plants and compared with chemically phosphorylated starches.

Therefore, the present invention also relates to starch which can be obtained from the plant cells, plants and/or propagation material according to the invention.

In one preferred embodiment of the invention, the starches according to the invention are characterised in that they have an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced peak temperature compared with starch from corresponding non-genetically modified wild-type plants.
The present invention also relates starch which is characterised in that it has an increased phosphate content in the C6 position of the glucose monomer and/or a modified phoshorylation pattern and/or an increased final viscosity and/or a reduced

peak temperature compared with starch from corresponding non-genetically modified wild-type plants.

The statements made in connection with the plant cells according to the invention are applicable with regard to the increase of the phosphate content in the C6 position, the modification of the phoshorylation pattern and the modification of the viscosity properties (final viscosity, peak temperature).

In a further embodiment, the invention relates to starches, preferably wheat starches, which are characterised in that in the C6 position of the glucose monomer they have a phosphate content of at least 0.1 nmol C6 P $mg^{-1}$ starch, particularly at least 0.5 nmol C6 P $mg^{-1}$starch, preferably at least 1 nmol C6 P $mg^{-1}$ starch, most preferably at least 2 nmol C6 P $mg^{-1}$ starch, most particularly at least 5 nmol C6 P $mg^{-1}$ starch, quite most preferably at least 10 nmol C6 P $mg^{-1}$ starch and/or an increased final viscosity and/or a reduced peak temperature, wherein in the C6 position of the glucose monomer the starch according to the invention has a phosphate content of 100 nmol C6 P $mg^{-1}$ starch at most, particularly 50 nmol C6 P $mg^{-1}$ starch at most, or 25 nmol C6 P $mg^{-1}$ starch at most.

In a further embodiment of the invention, the starches according to the invention can be characterised in that in the C6 position of the glucose monomer they have a phosphate content of at least 15 nmol C6 P $mg^{-1}$ starch, wherein in the C6 position of the glucose monomer the starch according to the invention has a phosphate content of 100 nmol C6 P $mg^{-1}$ starch at most, particularly 50 nmol C6 P $mg^{-1}$ starch at most, or 25 nmol C6 P $mg^{-1}$ starch at most.

In a further preferred embodiment, the starches according to the invention, preferably wheat starches, are characterised in that after conglutination the starches according to the invention form gels which exhibit an increased gel strength compared with gels of corresponding chemically phosphorylated starches of the same phosphate content and/or compared with gels of starches from corresponding non-genetically modified plants.

In a further preferred embodiment of the invention, the starches according to the invention can be characterised in that the major part of the phosphate groups are bonded to the amylopectin component of the starch, whilst the amylose only has a very low content of covalently bonded starch monophosphate esters. Compared with chemically phosphorylated starches of comparable C-6 phosphate content, the starches according to the invention from the plant cells according to the invention are distinguished in that the amylose of the starches according to the invention is less strongly phosphorylated than is the amylopectin component.

In a further embodiment of the invention, the starches according to the invention can be characterised in that the amylose component of said starch has a reduced total phosphate content in the amylose component compared with the amylose component of chemically phosphorylated starch, which preferably has the same phosphate content in the C6 position of the glucose monomer.

Methods of determining of the total phosphate content are known to one skilled in the art and are described below (see methods).

In a further embodiment the invention, the starch according to the invention can be characterised in that the total phosphate content, which can be detected by 31P NMR (Kasemsuwan and Jane (Cereal Chemistry 73 (6), (1996), 702-707) of the amylose component of said starch is reduced by at least 5%, preferably by at least 20%, particularly by at least 50% and most preferably by at least 80%, compared with the amylose component of chemically phosphorylated starch with the same phosphate content in the C6 position of the glucose monomer which is produced from starch of corresponding non-genetically modified plants.

In one preferred embodiment of the invention, the starches according to the invention can be characterised in that they exhibit a modified phoshorylation pattern compared with chemically phosphorylated starches.

The term "modified phoshorylation pattern" has already been defined in connection with the plant cells according to the invention.

In one preferred embodiment of the invention, the starches are starches from useful monocotyledon plants, such as rye-, barley, oats, wheat, millet, rice and maize starch. Wheat, rice and maize starch are preferred; wheat starch is particularly preferred.

The present invention further relates to a method of producing the starch according to the invention, comprising the steps of extracting the starch from a plant or plant cell as described above and/or from starch-storing parts of plant such as this and/or from the propagation material according to the invention. A method such as this also preferably comprises the step of harvesting the cultivated plants and/or starch-storing parts of said plants before the extraction of the starch, and most preferably further comprises the step of cultivating plants according to the invention and/or of propagation materials according to the invention before harvesting. Methods of extracting starch from plants or from starch-storing parts of plants are known to one skilled in the art. Methods of extracting starch from various starch-storing plants are also described, for example, in "Starch: Chemistry and Technology (edited by: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see Chapter XII, pages 412-468, for example: Maize and sorghum starches: production; by Watson; Chapter XIII, pages 469-479: Tapioca, arrowroot and sago starches: production; by Corbishley and Miller; Chapter XIV, pages 479-490: Potato starch: production and uses; by Mitch; Chapter XV, pages 491 to 506: Wheat starch: production, modification and uses; by Knight and Oson; and Chapter XVI, pages 507 to 528: Rice starch: production and uses; by Rohmer and Klem; and see Maize starch: Eckhoff et al., Cereal Chem. 73 (1996) 54-57. The extraction of maize starch on an industrial scale is generally effected by what is termed "wet milling"). Apparatuses which are customarily used for methods of extracting starch from plant material include mills, separators, decanters, hydrocyclones, spray driers and fluidised-bed driers.

The present invention also relates to starch according to the invention which can be obtained by the methods according to the invention which were described above.

The starches according to the invention can be subsequently modified by methods known to one skilled in the art, and in their unmodified or modified form are suitable for various uses in the food or non-food sectors.

Therefore, the present invention also relates to starches as defined according to the invention which have been subsequently chemically and/or physically modified.

Various options which can be used for chemical and/or physical modification are described in detail below.

The production of modified starches by means of genetic engineering operations on a plant can firstly modify the properties of the starch obtained from the plant so that further modification by means of chemical or physical methods no longer appear to be necessary. Secondly, starches which have been modified by genetic engineering methods can be subjected to further chemical and/or physical modifications, which results in additional improvements in quality for some of the areas of use described above. These chemical and physical modifications are known in principle. In particular, they comprise modifications by means of:

- heat treatment,
- treatment with acids,
- production of starch ethers,
  starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxylmethyl ethers, N-containing starch ethers, P containing starch ethers, S-containing starch ethers,
- production of crosslinked starches,
- production of starch graft polymers,
- oxidation, and
- esterification operations which are used for the production of phosphate, nitrate, sulphate, xanthate, acetate and citrate starches. Other organic acids can also be used for esterification.

In a further embodiment, the present invention relates to the use of the starches according to the invention in industry, preferably for the production of foodstuffs.

In a further embodiment, the invention relates to wheat flour which contains the starches according to the invention. Amongst its other properties, the wheat flour according to the invention is distinguished by modified baking properties compared with conventional wheat flours. Methods of producing wheat flour from wheat grains are known to one skilled in the art. By means of these methods, the wheat flour according to the invention can be isolated from the plant cells and plants according to the invention and from the propagation material according to the invention.

Thus the present invention also relates to wheat flour which can be obtained from the plant cells and plants according to the invention and from the propagation material according to the invention.

Compared with flours from corresponding non-genetically modified wild-type plants, the flours according to the invention are distinguished in particular by their enhanced water absorption capacity. Methods of determining water absorption capacity are known to one skilled in the art and are described in detail below (see methods).

In one preferred embodiment of the invention, the wheat flours exhibit a water absorption capacity which is increased by at least 5%, particularly by at least 15%, preferably by at least 20% and most preferably by at least 25% compared with wheat flours from corresponding non-genetically modified wheat plants.

Due to their high water absorption capacity, the flours according to the invention have the advantage for the industrial production of dough that less water has to be supplied to the flour during the dough preparation stage.
In a further embodiment, the present invention relates to the use of the wheat flour according to the invention and/or of the starch according to the invention for the production of a baking mixture and/or for the production of a food product.

In a further embodiment, the present invention relates the use of the wheat flour according to the invention and/or of the starch according to the invention for the coating of packages.

In a particularly preferred embodiment, the present invention relates to a baking mixture which contains the wheat flour according to the invention and/or the starch according to the invention. In this connection, the term "baking mixture" is to be understood to mean any mixture which can be used for the production of dough (e.g. yeast dough, pasta dough, shortcrust pastry, sour dough etc.) and/or of bakery products (e.g. bread, cakes, rolls, croissants, etc.).

In a further particularly preferred embodiment, the present invention relates to a food product, preferably bakery and dough products (e.g. pasta) which is produced using the wheat flour according to the invention and/or the baking mixture according to the invention and/or the starch according to the invention.

Compared with bakery products which are produced from wheat flours from corresponding non-genetically modified wheat wild-type wheat plants, the bakery products and/or food products according to the invention are distinguished on storage by a slowing down of the ageing process which the bakery products undergo.

The ageing process of a bakery product is closely linked to the degree of retrogradation (recrystallisation) of the amylopectin component of the starch. It is assumed that the ageing process of a bakery product and/or food product has progressed further, the higher is the degree of retrogradation of the amylopectin component The enthalpies of fusion of the amylopectin which can be determined by DSC provide information on the degree of retrogradation of the amylopectin. The degree of ageing of a bakery product can thus be determined by determining the enthalpy of fusion of the amylopectin at a given point in time by means of DSC (=differential scanning calorimetry) analysis (see methods).
If the enthalpy of fusion of the amylopectin of the bakery products and/or food products according to the invention is determined after storage for three and/or seven and/or thirteen days, for example, it is found that the enthalpy of fusion of the amylopectin is reduced compared with the enthalpy of fusion of the amylopectin of bakery products and/or food products which are stored for the same length of time

and which are based on wheat flours of corresponding non-genetically modified wild-type plants. This shows that the bakery products and food products according to the invention are distinguished by a slower ageing process compared with conventional products.

The Figures are as follows:

Figure 1: is a schematic illustration of an RVA profile;

Figure 2: is a plasmid map of the vector pUbi2afp

Figure 3: is a plasmid map of the vector pUbiR1

Figure 4: is a plasmid map of the vector p35SAcS (a derivative of pUC18 (Pietrzak M. et al., Nucleic Acids Res.14, (1986), 5857-5868): contains the pat gene from Streptomyces viridochromogenes (Wohlleben et al., Gene 70, (1988), 25-37) under the control of the CaMV35S promoter.

Figure 5: is a plasmid map of the vector pAct1-Fneo/Calgus (pUC19-derivative (Yannish-Perron et al., 1985, Gene 33: 103-119): formed from pAct1-Fneo (Müller et al., Plant Science 114, (1996), 71-82) and pCalgus, which contains the CaMV35S promoter (see US-A-5,352,605, for example), the AdhI intron from maize (Genes Dev 1987 Dec;1(10):1183-200) and the beta-glucuronidase (GUS) gene (The GUS Reporter System as a Tool to Study Plant Gene expression in: GUS Protocols: Using the GUS Gene as a Reporter of Gene expression, Academic Press (1992), 23-43).

Figure 6: shows the enthalpy of breadcrumbs during storage after 1, 3, 7 and 13 days.

Methods:

**Determination of the phosphate content in the C6 position of the glucose monomer (= C6 P content) of the starch (Nielsen et al. Plant Physiol. 105, (1994), 111-117):**

In order to determine the C6 P content of the starch, 50 mg starch were hydrolysed in 500 µl 0.7 M HCl for 4 hours at 95°C. The batches were subsequently centrifuged for 10 min at 15500 g and the supernatants were taken off. 7µl of the supernatants

were mixed with 193 µl imidazole buffer (100 mM imidazole, pH 7.4; 5 mM $MgCl_2$, 1 mM EDTA and 0.4 mM NAD). Measurements were made in a photometer at 340 nm. After establishing the base absorption, the enzyme reaction was initiated by adding 2U glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption was directly proportional to the concentration of the C-6 P content of the starch.

**Determination of the total phosphate content of the starch:**

Before determining the total phosphate content of the starch, the starch had to be completely separated from phosphorylated non-glucans such as phospholipids. The Determination of the total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118), as follows:
About 50 mg starch were treated with 30 µl of 10% ethanolic magnesium nitrate solution and were ignited for three hours in a muffle furnace. The residue was treated with 500 µl 0.5 M hydrochloric acid and incubated for 30 min at 60°C. A 20 µl aliquot was then made up to 300 µl with 0,5 M hydrochloric acid, was added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 1 M sulphuric acid, and was incubated for 20 min at 45°C. This was followed by a photometric determination at 820nm using a phosphate calibration series as a standard.

**Determination of the gel strength (texture analyser):**

2.5 g starch (TS) were conglutinated in 25 ml $H_2O$ in an RVA instrument (see determination of the viscosity properties by means of a Rapid Visco Analyzer (RVA)) and were subsequently stored for 24 hours at room temperature. The samples were fixed under the sensor (a cylindrical plunger with a planar surface) of a TA-XT2 texture analysers supplied by Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following instrument settings:

- Test speed 0.5 mm/s
- Depth of penetration 7 mm
- Contact area 113 $mm^2$
- Pressure 2g

**Determination of the viscosity properties (e.g. final viscosity) by means of a Rapid Visco Analyzer (RVA):**

2.5 g starch (dry weight) were taken up in 25 ml $H_2O$ and used for analysis in a Rapid Visco Analyzer (Newport Scientific Pty Ltd., Investment Support Group, Warriewod NSW 2102, Australia). The instrument was operated according to the manufacturer's instructions. In order to determine the viscosity of the aqueous solution of the starch, the starch suspension was first heated for one minute to a temperature of 50°C and was then heated from 50°C to 95°C at a rate of 12°C per minute. The temperature was subsequently held for 2.5 minutes at 95°C. Thereafter, the solution was cooled from 95°C to 50°C at a rate of 12°C per minute. Finally, the temperature was held for a further 6 minutes at 50°C. The viscosity was determined over the entire duration of the test.

**Isolation of the amylose component of the starch by aqueous leaching:**

It is known that amylose can be obtained from starch by aqueous leaching (Roger and Colonna (International Journal of Biological Macromolecules 19, (1996), 51-61). In orde to isolate the amylose component of the starch, 25ml of an aqueous starch suspension (10% w/v) were prepared and heated in an RVA (Newport Scientific) with stirring, using the following profile:

| Time [min] | Type | Value [°C] or [rpm] |
|---|---|---|
| 0 | Temp | 30° |
| 0 | Speed | 960 rpm |
| 0.5 | Speed | 300 rpm |
| 10 | Temp | 95° |

Directly after the viscosity of the suspension or solution exceeded 200 cP, the programme was terminated and the starch suspension or solution was transferred into a cold, 50ml reaction vessel, mixed with 25ml of cold water and cooled for 10 min in an ice bath to about room temperature (RT). Thereafter, the undissolved glucan was formed into a pellet by centrifugation (20min at 2300g at RT) and the clear supernatant was treated with 10mg NaCl/ml. The dissolved glucan was then precipitated on to ice from 80% ethanol, was washed with ethanol again, and was dried for 3 days at 37°C. The pellet was subsequently ground (15s 30Hz with

tungsten carbide balls in a in a vibrating mill supplied by Retsch, Germany), and 25mg of the pellet was suspended in 500µl of 0.7M HCl hydrolysed at 95 °C for 4 hours. Undissolved particles were then removed by centrifugation and the content of glucose-6P was determined enzymatically (see above: "Determination of the phosphate content in the C6 position of the glucose monomer").

**Procedure for the small scale water absorption test on wheat flour:**

Materials

Eppendorf cups (2 ml) with screw cap from Sarsted.

Eppendorf centrifuge (Sigma 202 )

Vortex (Wilten)

Analytical Balance (Mettler)

Method

Measure the weight of the epp.cup to four decimal places using the analytical balance.

Fill the cup with 100 to 110 mg flour and note the exact weight.

Put the cup with flour on the vortex and add 1.0 ml water during vortexing.

Keep on vortexing for 10 seconds.

Add 0.9 ml water and close the cup with the screw cap.

Wait ten minutes.

Put the cups in the 6*10 rotor (swing out) in the centrifuge.

Run the centrifuge for 30 minutes at 10,000 rpm (about 8600*g).

Pour off the water from the cup.

Dry the inside of the cup with filter paper without disturbing the wet residue.

Measure the weight of the Eppendorf cup with the wet flour to four decimal places using the analytical balance.

Calculate the weight of the absorbed water.

Determine the waterabsorption of each flour ten times and calculate the average.

**Differential Scanning Calorimetry :**

Differential Scanning Calorimetry (DSC) is a technique studying thermal transitions, in this case of breadcrumbs.

With this technique the heat flow into a sample is determined at a continuous (relatively low) heating rate. Either a peak (offset from the base line (endotherm)) or a step in the base line (glass transition) determines transitions.

Integration of the peak area with respect to the base line gives the enthalpy of the transition. For breadcrumbs, an amylopectin heat of fusion between 40 and 70°C (60°C peak) and an amylose-lipid decomplexation at about 120°C are generally observed.

Differences in the heat of fusion of amylopectin between the various flour samples indicate differences in retrogradation behaviour, and hence indicate that the bread is becoming stale. The enthalpy of fusion of the amylopectin crystals increases with storage time after baking. An increase in the enthalpy of fusion during storage is a measure of an increase in the amount of recrystallised amylopectin with time. The enthalpies (j/g) were calculated on a dry matter basis in order to correct for differences in moisture content.

DSC measurements were made using a TA Instruments Type 2920 calorimeter with LNCA cooling unit. This cooling unit uses liquid nitrogen for fast cooling after the experiment.

Samples (40 - 50 mg breadcrumbs) were placed in a DSC pan and compressed by hand with a nail. After sealing the DSC pan with a compression unit the sample was placed in the DSC-apparatus.

Samples (40 - 50 mg breadcrumbs) were heated from 20°C to 160°C at a rate of 7.5 C/min in stainless steel hermetically sealed pans (Perkin Elmer). A medium pressure pan (120µl volume), filled with 100 mg aluminium, was used as a reference.

The enthalpies of the crumbs of the mini-loaves were measured after 1, 3, 7 and 14 days of storage in single measurements.

The following examples serve to explain the invention without limiting it in any respect:

Example 1

**Production of vector pUbiR1 for the transformation of wheat plants**

In order to produce vector pUbiR1, vector pUbi.cas was first produced as follows: Vector pUbi2afp (see Figure 2) was partially cut with restriction enzymes PstI/EcoRI. The 4.19 Kb fragment which resulted therefrom, consisting of the pUC19 vector (Yannish-Perron et al., 1985, Gene 33: 103-119), the 1.5 Kb ubiquitin promoter and of the first exon of the ubi gene and a shortened ubiquitin1 intron (ClaI deletion) (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689) was used further after gel elution.

The nos terminator was isolated as a PstI/EcoRI fragment from the vector pAct.cas (Product Number Cambia TG0063, supplied by Cambia, GPO Box 3200, Canberra ACT 2601, Australia) and ligation of the two fragments was effected to give the vector pUbi.cas.

The cDNA of the potato R1 gene (SEQ ID No.1) was subsequently isolated as a partial digest (SmaI/Asp718 fragment) from the vector pRL2 (WO97/11188, Example 4, deposited at the German Collection for Microorganisms with the number DSM 10225) and was integrated in the vector pUbi.cas (restriction with SmaI/Asp718). The resulting construct was designated as pUbiR1 (see Figure 3). This construct comprises the coding region of the R1 cDNA from potatoes, which is under the control of the ubiquitin promoter (Christensen et al., 1992, Plant Mol. Biol. 18: 675-689), and also comprises, as additional regulation units, the first exon of the ubi gene (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689) and the shortened first intron (Christensen et al., Plant Mol. Biol. 18, (1992), 675-689), the internal ClaI fragment being deleted).

Example 2

**Production of wheat plants which express an R1 gene from Solanum tuberosum, and analysis of the phosphate content of the starch of these plants**

The biolistic transformation method was used for the transformation of wheat (Becker et al., Plant J. 5 (2), (1994), 229-307). The vector pUbiR1 and, for biolistic cotransformation, the vectors p35SAcS (see Fig. 4) or pAct1-Fneo/Calgus (Müller et al., Plant Science 114, (1996), 71-82, see Fig. 5) were used in identical molar ratios in the DNA particle precipitation batch.

Vector p35SAcS was produced as follows: The pat gene from S. *viridochromogenes* (Wohlleben et al., Gene 70, (1988), 25-37) was amplified via a polymerase chain reaction. The primers used were designed so that a *Bam*HI cleavage site was created on both sides of the amplificate. The *Bam*HI fragment was subsequently cloned into the *Bam*HI cleavage site placed between the 35S promoter and the 35S terminator of the vector pDH51 (Pietzrak et al., Nucleic Acids Res. 14, (1986), 5857-5868). The cassette containing the 35S promoter, the *pat* gene and the 35S terminator was cut as an EcoRI fragment and was cloned into in the EcoRI cleavage site of the vector pUC18 (Pietzrak et al., Nucleic Acids Res. 14, (1986), 5857-5868). Scutelli from 14-day old, unripe embryos of wheat plants were used as target cells for transformation. Transformation was followed by *in vitro* culture on $MS^-$ medium (PCT/EP97/02793) containing 2 mg/l 2,4-D (=2,4-dichlorophenoxyacetic acid). Two weeks after transformation, subculture was effected on the same medium, to which 2 mg/l phosphinotricin or 150 mg/l kanamycin sulphate had been added. After a further two weeks, the developing calli were transplanted on to a regeneration medium ($MS^-$ medium with 0.1 mg/l 2,4-D and 2 mg/l PPT or 150 mg/l kanamycin). The developing shoots were transferred to semi-concentrated $MS^-$ medium without 2,4-D and phosphinotricin or kanamycin and were subsequently transferred into the ground. About 14 days after their establishment in the ground, the transgenic plants were identified by spraying twice with an aqueous solution containing 150 and 200 mg/l phosphinotricin, respectively, 0.1% Tween 20 (ICI America, corresponding to polysorbate 20) or by spraying twice with 2.5% kanamycin sulphate, 0.1% Tween 20.

Expression of the potato R1 gene in $T_0$ plants

Expression of the potato R1 gene in transgenic wheat $T_0$ plants was detected by Northern and Western blotting analyses and by the enzymatic determination of the

phosphate content in the C6 position of the glucose monomer of the starch from caryopses (Nielsen et al., Plant Physiol. 105, (1994), 111-117).

R1 protein was detected in the transgenic wheat plants with the aid of an anti-potato R1 protein antibody (Ritte et al., Plant J. 21(4), (2000), 387-391).

Protein extracts from the endosperm of unripe caryopses about 20 days old was used for screening the transgenic plants.

Starch from transgenic $T_0$ plants was isolated from unripe and ripe wheat caryopses in order to determine C6 phosphate. The caryopses were triturated in a mortar to form a powder. After adding 15 ml of 100 mM Tris buffer, pH 8.0, the suspension was filtered through a 100 µm sieve and the starch was pelletised by centrifugation (2600 g, 5 min, 4°C). The supernatant was discarded. The starch pellet was subsequently re-suspended in 2 ml of 100 mM Tris buffer, pH 8.0 and transferred to an 8 ml Percoll gradient. The starch was palletised by centrifugation for 15 minutes at 170 g and 4°C. The starch pellet was subsequently washed three times with 10 ml of 100 Tris buffer, pH 8.0. Finally, the starch was degreased by acetone incubation and was dried.

The C6 P content was determined by glucose-6-phosphate determination by means of an optical-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) in the manner described above.

The tests showed that of the wheat $T_0$ plants which gave a positive result in Southern blotting analysis about 50% of the lines in the caryopses synthesised a starch which had an increased phosphate content in the C6 position of the glucose monomer compared with starch from corresponding non-genetically modified wild-type-plants of the Florida variety. Table 1 gives the data for some selected lines.

Analysis of natural descendants

Seeds which were obtained naturally from R1-expressing parental lines was sown and the segregation ratios were determined. It was possible to detect both the integration and also the expression of the R1 gene in $T_1$descendants of different lines by Southern and Northern blotting analyses. The phosphate content of these lines in the C6 position of the starch was determined. Table 1 shows the data for some selected lines.

Table 1:

| Line No. | C6 P in nmol/mg | Gene-ration | Standard deviation |
|---|---|---|---|
| Wild-type Florida variety | Not detectable | - | 0.0 |
| 19 | 2.8 | T0 | 0.2 |
| 20-25 | 6.7 | T1 | 0.2 |
| 37 | 1.4 | T0 | 0.3 |
| 40A-11-8 | 10.7 | T2 | 0.7 |

Example 3

**RVA analysis of the starch of plants from transgenic wheat plants which express an R1 gene from Solanum tuberosum, and investigation of the gel strength**

The starches from the wheat plants described in Examples 1 and 2 were subjected to an RVA analysis.

The results of the RVA analysis (experimental: see methods) showed that the viscosity behaviour of the starches from the transgenic wheat plants which express an R1 gene from Solanum tuberosum is significantly modified compared with starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) (see Table 2).

Table 2:

| Line No. | RVA Max (%) | RVA Min (%) | RVA Fin (%) | RVA Set (%) | RVA T (%) |
|---|---|---|---|---|---|
| Wild-type (Florida) | 100 | 100 | 100 | 100 | 100 |
| 19 (T0) | 98.7 | 113.8 | 116.8 | 120.7 | 102 |
| 20-25 (T1) | 155.3 | 190 | 193.8 | 198.8 | 99 |
| 37 (T0) | 143.8 | 156.2 | 148.8 | 139 | 101 |
| 40-11-8 (T2) | 156.2 | 185.5 | 187.7 | 190.6 | 97 |

Legend:

RVA = Rapid Visco Analyzer

Max = see Figure 1

Min = see Figure 1

Fin = final viscosity, see Figure 1

Set = Setback = Difference between Min and Fin, see Figure 1

T = Conglutination temperature, see Figure 1

The percentages are given with respect to the wild-type (= 100 %).

The gel strengths of the starches (determination of the gel strength: see methods) from the transgenic wheat plants which express an R1 gene from Solanum tuberosum differed both from those of starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) and from those of wild-type-starches (Florida variety), which had subsequently been chemically phosphorylated (see Table 3).

In contrast to chemically phosphorylated starches with a comparable phosphate content in the C6 position of the glucose monomer, after conglutination the gels of the starches according to the invention exhibited an increased gel strength compared with gels of starches of wild-type-plants. In contrast to this, the chemically phosphorylated starches which had been produced by the method described by Lim compared with gels of starches of wild-type plants.

Table 3:

| Line No. | Gel strength (TA) (%) | Phosphate content in the C6 position in µmol phosphate/g starch |
|---|---|---|
| Wild-type (Florida) | 100 | Not detectable |
| 19 (T0) | 167 | 2.8 |
| 20-25 (T1) | 192 | 6.7 |
| 37 (T0) | 168 | 1.4 |
| 40-11-8 (T2) | 224 | 10.7 |
| | | |
| Chemically phosphorylated wild-type (Florida) starch | | |
| STMP1 | 84 | 2.1 |
| STMP6 | 75 | 6.5 |
| STMP8 | 67 | 11.5 |

TA = texture analyzer

The percentages are given with respect to the wild-type (= 100 %).

Example 4

**DSC analysis of the starch of plants from transgenic wheat plants which express an R1 gene from Solanum tuberosum**

The starches from the wheat plants described in Examples 1 and 2 were subjected to DSC analysis.

The peak temperature was determined using an instrument supplied by Perkin Elmer (instrument designation: DSC-7) using large volume capsules, wherein the sample to be investigated had a ratio of starch to total water content of about 1:4 and measurements were made over the temperature range from 10°C to 160°C at a heating rate of 10°C/min.

The results of the DSC analysis (Table 4) show that the peak temperature of the starches from the transgenic wheat plants which express an R1 gene from Solanum tuberosum exhibit a reduced peak temperature Tp compared with starches from corresponding non-genetically modified wild-type-wheat plants (Florida variety) and also compared with starches of comparable phosphate content in the C6 position of the glucose monomer which were subsequently chemically phosphorylated.

Table 4:

| Sample | Water content of sample (%) | Starch (dry weight): water (total water content) | Peak temperature in °C (first peak maximum) | Other peak maxima in °C |
|---|---|---|---|---|
| Wild-type (Florida) | 16.6 | 1 : 4.01 | 61 | 101 |
| 19 (T0) | 10.5 | 1 : 3.99 | 58 | 100 |
| 20-25 (T1) | 16.9 | 1 : 4.05 | 55 | 100 |
| 40-11-8 (T2) | 16.9 | 1 : 3.98 | 56 | 100 |
| STMP2* | 10.9 | 1 : 4.00 | 60 | 101 |
| STMP6 | 10.7 | 1 : 4.00 | 61 | 101 |

* The chemically phosphorylated starch STMP2 had a phosphate content in the C6 position of 3.0 µmol phosphate/g starch. All the other phosphate data are given in Table 3.

Example 5

**Determination of the C6 P content of the amylose component of various starches**

Apart from starch from transgenic wheat plants which express an R1 gene from Solanum tuberosum, two chemically phosphorylated starches (STMP3 and STPP4) were investigated. Chemical phoshorylation was effected by the method described by Lim and Seib (Cereal Chem. 70 (2), (1993), 137-144), wherein STMP3 was produced by reacting the starch for 2 hours at pH 6, and STPP4 was produced by reaction for 30 minutes at pH 8. The abbreviation STMP represents the selected phoshorylation reagent, namely sodium trimetaphosphate; STPP represents sodium tripolyphosphate.

The amylose component of various starches was isolated as described above (isolation of the amylose component of the starch by aqueous leaching).
The phosphate content in the C6 position was determined as described above (determination of the phosphate content in the C6 position of the glucose monomer).

Table 5:

| Sample | C6 P content in µmol phosphate/g starch | C6 P content in µmol phosphate/g amylose | C6 P content of amylose/ C6 P content of starch |
|---|---|---|---|
| 40-11-8 (T2) | 10.7 | 1.9 | 0.18 |
| STPP4 | 20.5 | 20.4 | 0.99 |
| STMP3 | 6.0 | 5.1 | 0.85 |

It can be seen that the ratio of the C6 P content of the amylose to the C6 P content of the starch in the chemically phosphorylated starches is considerably higher than in the starches from the transgenic wheat plants which superexpress an R1 Gen from potatoes. The chemically phosphorylated starches have a higher content of phosphorylated amylose than do the starches from the transgenic wheat plants.

Example 6

**DSC analysis of breadcrumbs from micro-loaves produced from flour of different origins**

Wheat flour from wheat wild-type plants (Florida variety) and from genetically modified wheat plants which superexpress the R1 gene from potato was used to produce micro-loaves, using the following recipe:

| | |
|---|---|
| Wheat flour | 10.00 g |
| Yeast | 0.25 g |
| Salt | 0.20 g |
| Dextrose | 0.10 g |
| Ascorbic acid | 20 ppm |
| Water | the amount of water as determined in the water absorption test and corrected for consistency (see method "Procedure for small scale water absorption test on wheat flour") |

The dough of the genetically modified wheat plants became very sticky during the mixing process. To obtain doughs with correct handling properties a smaller amount of water was used. The dough consistency was corrected by technical sensory perception of the stiffness of the dough.

The dough was proofed in a proofing cabinet for 45 minutes (28°C), manually worked through, proofed for 15 minutes (28°C) for a second time, formed and put in a baking tin. Subsequently, the dough was given a final proof for 60 minutes (28°C). The loaves were baked in an oven for 15 minutes (200°C), with the tins embedded in a moist wooden frame. This embedding was done to shield the baking tin from high amounts of radiative heat. Finally, the loaves were baked for 5 minutes, without the wooden frame, to allow the crust to brown.

Differential scanning calorimetry (DSC) (see methods) was used to determine thermal transitions of the breadcrumbs. The enthalpies of crumbs from the loaves were measured after 1, 3, 7 and after 14 days of storage (Figure 6).

Figure 6 shows the enthalpy of the amylopectin fusion peak (40°C-70°C) for stored bread crumbs after 1, 3, 7 and 13 days of storage, giving an indication of staling effects. The breadcrumbs from loaves made from wild-type (Florida) flour have a higher enthalpy compared with breadcrumbs of the same age made from flour from two different lines (GMO1 and GMO2) of genetically modified wheat plants which

superexpress the R1 gene from potato. This indicates a lower level of recrystallised starch, less retrogradation of starch and less staling of the bread.

Sequence protocol:

SEQ ID No. 1 (R1 cDNA potato, nucleotide sequence 5061 bp, coding region: bp 216 to bp 4607):

gaattgtaatacgactcactatagggcgaattgggtaccgggccccccctcgaggtcgacggtatcgataagcttgat
atcgaattcgcggccgcttttgcttcgtgaattcatcttcatcgaatttctcgacgcttcttcgctaatttcctcgttacttcacta
gaaatcgacgtttctagctgaacttgagtgaattaagccagtgggaggatatgagtaattccttagggaataacttgctg
taccagggattcctaacctcaacagtgttggaacataaaagtagaatcagtcctccttgtgttggaggcaattctttgtttc
aacaacaagtgatctcgaaatcacctttatcaactgagtttcgaggtaacaggttaaaggtgcagaaaaagaaaata
cctatgggaaagaaccgtgctttttctagttctcctcatgctgtacttaccactgatacctcttctgagctagcagaaaagtt
cagtctagaagggaatattgagctacaggttgatgttaggcctcccacttcaggtgatgtgtcctttgtggattttcaagct
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SEQ ID No.2 (R1 protein from potato, amino acid sequence: 1464 amino acids)

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